

## Antimicrobial effectiveness and color stability of protein-based films incorporated with essential oils

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### Abstract

The incorporation of essential oils (EO) into edible active films can protect consumers' health by reducing food poisoning of microbial origin. Packaging attributes such as color and flavor can be determinant factors in packaged food products. The objective of this study was to evaluate the antimicrobial activity and color stability of protein-based films incorporated with cinnamon and rosemary EOs. The agar diffusion method was used to determine the antimicrobial effectiveness of the film against the microorganisms *Escherichia coli*, *Staphylococcus aureus*, and *Penicillium* spp. The film incorporated with cinnamon cassia EO showed the best results against the microorganisms tested while, film incorporated with rosemary EO not exhibited antimicrobial activity. Great changes in instrumental color were observed in the films over time. Biopolymeric films with the incorporation of cinnamon EO have proven to be promising alternatives for improving quality and food safety. It has potential application to wrap food reducing microbial contamination.

### Keywords

Cinnamon

Rosemary

Active packaging

Oxidation

Whey protein

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### Introduction

Several studies have been focused on new packaging technologies such as active packaging (Janjarasskul *et al.*, 2016; Dicastillo *et al.*, 2016; Rizzolo *et al.*, 2016). Various natural additives can be incorporated into the packaging material, arousing great interest by food industries once they can replace synthetic agents (Bolumar *et al.*, 2016; Dicastillo *et al.*, 2016). The trend is getting closer to natural foods, thus minimizing environmental impacts and extending the product's shelf life. In this regard, biopolymer-based films for use as packaging materials have gained prominence, especially as different active agents can be incorporated into the films (Seydim and Sariku, 2006; Soazo *et al.*, 2011; Bahram *et al.*, 2014).

Whey protein-based films are clear, odorless, and have excellent barrier properties with respect to oxygen and lipids (Krochta, 2002; Perez-Gago *et al.*, 2006; Ramos *et al.*, 2013). These films are biodegradable and meet the environmental appeal, since they are originated from renewable sources and agro-industrial waste. Furthermore, they can be carriers of various antimicrobial agents, including essential oils (Bahram *et al.*, 2014; Fernández-Pan *et al.*, 2014).

Essential oils (EOs) can be extracted from different plant parts, and present a variety of phenolic compounds and terpenes, which exhibited antioxidant and antimicrobial activities, besides being ingredients with flavoring properties (Bouaziz *et al.*, 2009; Wannan *et al.*, 2010; Hossain *et al.*, 2012; Riahi *et al.*, 2013). Rosemary and cinnamon EOs have attracted great interest from scientific community due to be considered a natural product with numerous benefits for human health. Scientific evidence shown antiproliferative activity, analgesic, antianxiety, anti-inflammatory cytotoxic and apoptotic effects (Unlu *et al.*, 2010; Ribeiro –Santos *et al.*, 2015). In the food industry rosemary and cinnamon may be used as flavoring, antimicrobial and antioxidant agents (Jayaprakasha *et al.*, 2007; Ribeiro –Santos *et al.*, 2015).

Considering that the incorporation of EO into active films may affect color, aroma, and other properties of the processed product, they need to be used with caution in specific food products. Given the importance of packaging in the natural products market for extending product's shelf life, this study aimed to evaluate the antimicrobial activity and optical stability of whey protein-based films incorporated with cinnamon and rosemary EOs.

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## Materials and Methods

### Whey protein

Whey protein concentrate (WPC, 82.8% protein) was purchased from Protosa – Proteins and Nutritionals (Glanbia Nutritionals, EUA), with certificate of analysis sent by the supplier.

### Essential oil (EO)

The following EOs were commercially purchased (Ferquima Indústria e Comércio Ltda, São Paulo, Brazil): Rosemary EO (*Rosmarinus officinalis* L) originated from Tunisia, obtained by steam distillation of rosemary leaves and presenting 1.8 cineol,  $\alpha$ -pinene, and camphor as major components; Cinnamon cassia EO (*Cinnamomum cassia* (L.) J. Presl) originated from China, obtained by steam distillation of leaves, stems and hulls, having cinnamaldehyde as a major component; Cinnamon EO (*Cinnamomum zeylanicum* Blume) originated from China, obtained by steam distillation of cinnamon leaves, having eugenol as a major component.

### Manufacture of the edible films

The protein-based films were obtained by the casting method, according to Bahram *et al.* (2014), with modifications. Whey protein (8% w/w) was dispersed in distilled water and homogenized until complete solubilization. The pH of the filmogenic solution was adjusted to 7.0 with 5N and 1N sodium hydroxide. The solution was heated in water bath (Quimis, Q214M, São Paulo) at 80°C for 30 min to protein denaturation, and cooled in an ice bath to room temperature (25°C). After cooling, glycerol (8%, w/w) was added and the mixture homogenized briefly, and 2.7% (w/w) EO was added. The solution was stirred in Ultra Turrax (T10 Basic, USA) at 14000 rpm, and 15 mL were dispersed in tetrafluoroethylene plate (Teflon®) for drying at room temperature (25°C) for 48 h. A negative control film without addition of EO was prepared for comparison purposes.

The thickness of the films was measured in 9 different points with the aid of a digital micrometer 0-25 mm (Digimes Instrumentos de Precisão Ltda. Mooca, São Paulo). The films were stored under light, in low density polyethylene plastic containers, with a thickness of 0.094  $\mu\text{m}$  each side.

### Antimicrobial activity of the active films

The antimicrobial activity test was determined for the following microorganisms: *Escherichia coli* (ATCC 1122), *Staphylococcus aureus* (ATCC 6538), and *Penicillium* spp. For the bacterial suspension, the microorganisms were cultivated in Brain Heart

Infusion Broth (BHI), and inoculated in Plate Count Agar (PCA) and Potato Dextrose Agar (PDA) for bacteria and fungi counts, respectively.

The antimicrobial effectiveness of the films was evaluated *in vitro* by diffusion technique in agar (CLSI, 2003). Discs of films with 0.6 cm in diameter were exposed to UV light for 15 min for sterilization, and placed at the center of the petri dish containing the solid medium (Mueller-Hinton) previously inoculated with 0.1 mL of a bacterial suspension containing  $10^8$  CFU  $\text{mL}^{-1}$  using the McFarland 0.5 standard or a fungal suspension containing  $10^8$  spores  $\text{mL}^{-1}$  in a Neubauer chamber. Then, the plates were incubated in a germination chamber at 36°C for 24 hours and 25°C for 48 hours, for growth of bacteria and fungi, respectively, and the formation of inhibition zone around the film was evaluated. All tests were performed in triplicate.

### Optical property and stability of the films

Color measurements were performed in a colorimeter (spectrophotometer CM-5, Konica Minolta, Osaka, Japan), by assessing the color parameters brightness ( $L^*$ ),  $a^*$  coordinate representing redness (+a) or green (-a), and  $b^*$  coordinate representing yellow (+b) or blue (-b), using D65 (illuminant) and 10° (observation angle) at time zero (after preparing the film) and after 15 and 30 days of storage. Three samples of each film were analyzed in triplicate.

### Statistical analysis

A completely randomized experimental design was used, in triplicate, with two replications. Tukey's test was performed to compare the means of the antimicrobial results ( $p < 0.1$ ) and optical stability ( $p < 0.05$ ), using software Statistica® 10.0 (Statsoft Inc. 2325, Tulsa, OK).

## Results and Discussion

### Active films incorporated with essential oils

The presence of the EO in whey protein based film allowed that the films incorporated with EOs detached more easily from the plate when compared to the films without addition of EO (control). All films were transparent, uniform, flexible, and bright, and exhibited smooth surface with no bubbles visible. Different colors and aromas were observed depending on the EO used. The films incorporated with cinnamon and rosemary EO showed characteristic aroma, which was not observed for the control film.

The film thickness increased slightly with the addition of EO, with values of 0.182 mm and 0.202

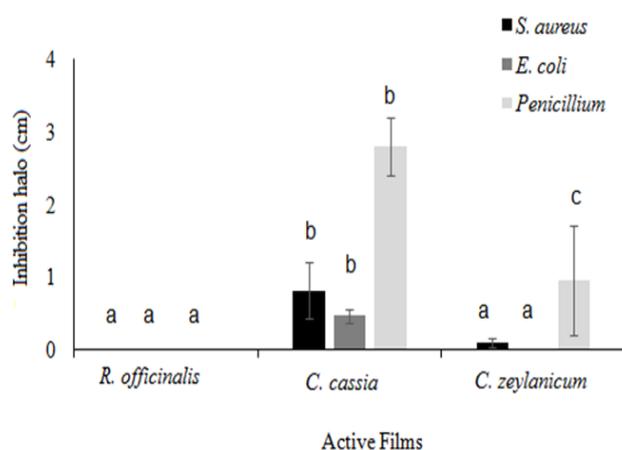


Figure 1. Antimicrobial activity of the films incorporated with *Rosmarinus officinalis*, *Cinnamomum cassia*, and *Cinnamomum zeylanicum* EO, against the microorganisms *Staphylococcus aureus*, *Escherichia coli* and *Penicillium* spp. Different letters show significant differences ( $p < 0.1$ ) between the means of halos for the same microorganism by the Tukey's test. The inhibition halo does not include the film disc diameter.

$\pm 0.003$  mm for the control (without EO) and active films, respectively.

All films had distinctly different appearance at both sides. No brightness was observed at the front side of the plate, whereas the other side was bright. Difference at both sides was found also by Ramos *et al.* (2013) in the appearance of whey protein-based films done in plastic Petri dishes, probably due to phase separation in the filmogenic solution during drying, rather than the material of the plate (Ramos *et al.*, 2013).

#### Antimicrobial activity of the active films

The results of the antimicrobial activity of all films are presented in Figure 1. Although whey protein based-films without incorporation of EO were used as control to determine possible intrinsic antimicrobial effects, no inhibition zone was observed against the three microorganisms studied, as expected. The different inhibitory effects of whey protein-based films are due to the biological compounds of the EO incorporated into the polymer matrix, which in turn are affected by several factors, including the variety of plant or plant part used for oil extraction (Khajeh *et al.*, 2005; Elzaawely *et al.*, 2007). The film incorporated with EO extracted from *C. cassia* had greater antimicrobial activity when compared to the other films, while the film incorporated with rosemary EO had no effect against the microorganisms tested (Figure 1).

*C. cassia* and *C. zeylanicum* contain cinnamaldehyde and eugenol, respectively as the major component. According to Singh *et al.*

(2007) and Dussault *et al.* (2014), eugenol has lower antimicrobial effect when compared to cinnamaldehyde, which may explain the difference in antimicrobial activity between different cinnamon species. Similarly, Seydim and Sarikus (2006) found that films incorporated with up to 4% rosemary EO exhibited no antimicrobial effect against *S. aureus*. In addition, several authors have reported that high levels of rosemary EO are required to provide an antimicrobial effect (Seydim and Sarikus, 2006; Romano *et al.*, 2009; Mathlouthi *et al.*, 2012; Ojeda-Sana *et al.*, 2013).

However, Fernández-Pan *et al.* (2012) have reported that the low antimicrobial activity of the films can be due to the EOs do not reach the minimum concentration required for microbial inhibition in the film matrix, since the oil is more diluted, thus a higher concentration is required. Thus, the minimum inhibitory concentration (MIC) of a given EO can be higher than the levels to be incorporated into the polymeric matrix, and the maximum EO level that can be incorporated into the WPC matrix depends on the EO used (Fernández-Pan *et al.*, 2012). Different types of microorganisms and their numbers in the culture medium may interfere with the antimicrobial effect of the active films. The *E. coli* bacteria were more resistant than *S. aureus* and *Penicillium* spp, probably due to the external lipopolysaccharide component of the outer membrane, which surrounds the peptidoglycan layer (Zinoviadou *et al.*, 2009).

#### Optical stability

The color of packages is an important attribute because it directly affects consumers' acceptability. Table 1 shows the results of optical stability of the films with respect to the luminosity and chroma coordinates ( $a^*$  and  $b^*$ ). At time zero, all films were clear, with similar brightness indexes ( $L^*$ ) when compared to the control film, which ranged from 91.82 to 94.80. Both the control film and the film incorporated with rosemary EO were less yellowish than the films incorporated with cinnamon EO. The film incorporated with EO extracted from *C. zeylanicum* was the most reddish (higher  $a^*$  value), whereas the films containing *C. cassia* EO were more yellowish (higher  $b^*$  value) and less reddish (lower  $a^*$  value).

At days 15 and 30, despite no significant differences ( $p < 0.5$ ) were observed for both  $L^*$  and  $a^*$  values in the films incorporated with rosemary EO when compared to the control film, a significant difference ( $p > 0.5$ ) was observed for the coordinate  $b^*$  at day 15. The films incorporated with cinnamon EO presented significant differences ( $p > 0.5$ ) in color

Table 1. Color parameters L\* (lightness), a (red - green) and b (yellow - blue) for WPC based films incorporated with different essential oils in the different storage time

Time (Days)	Film	Lightness (L)	Red - green (a)	Yellow - blue (b)
0	Control	94.80 <sup>A,ab</sup>	-0.37 <sup>A,ab</sup>	8.34 <sup>A,a</sup>
	<i>Rosmarinus officinalis</i>	96.68 <sup>A,b</sup>	-0.68 <sup>A,a</sup>	7.51 <sup>A,a</sup>
	<i>Cinnamomum cassia</i>	91.82 <sup>A,a</sup>	-9.12 <sup>A,c</sup>	65.49 <sup>A,b</sup>
	<i>Cinnamomum zeylanicum</i>	92.22 <sup>A,a</sup>	1.56 <sup>A,b</sup>	39.71 <sup>A,c</sup>
15	Control	94.77 <sup>A,a</sup>	-4.25 <sup>B,a</sup>	21.27 <sup>AB,a</sup>
	<i>Rosmarinus officinalis</i>	95.66 <sup>A,a</sup>	-2.03 <sup>B,a</sup>	13.33 <sup>B,b</sup>
	<i>Cinnamomum cassia</i>	80.10 <sup>B,b</sup>	8.78 <sup>B,b</sup>	101.43 <sup>B,c</sup>
	<i>Cinnamomum zeylanicum</i>	89.02 <sup>B,c</sup>	2.49 <sup>B,c</sup>	49.88 <sup>A,d</sup>
30	Control	93.90 <sup>B,a</sup>	-5.02 <sup>B,a</sup>	27.35 <sup>B,a</sup>
	<i>Rosmarinus officinalis</i>	95.02 <sup>A,a</sup>	-2.99 <sup>C,a</sup>	17.58 <sup>C,a</sup>
	<i>Cinnamomum cassia</i>	74.75 <sup>C,b</sup>	16.81 <sup>C,b</sup>	104.5 <sup>B,b</sup>
	<i>Cinnamomum zeylanicum</i>	89.92 <sup>AB,c</sup>	1.68 <sup>AB,c</sup>	47.02 <sup>A,c</sup>

Different superscript letters are significantly different ( $p < 0.05$ ) by Tukey's test. Different low-case letters in the same storage time indicate significant differences ( $p < 0.05$ ) between the films. Different capital letters in the same films in different storage time indicate significant differences ( $p < 0.05$ ). Control film = without addition of essential oil.

when compared to the other films. Although the film incorporated with rosemary EO presented similar color to the control film, with a stable color and with no significant differences ( $p > 0.5$ ) in brightness throughout 30 days, it was less reddish and more yellowish over time. Major changes were observed for the film incorporated with *C. cassia* EO, with L\* values ranging from 91.82 to 74.75 after 30 days. A significant difference ( $p > 0.5$ ) was observed for the coordinate a\*, which changed from greenish to reddish (-9.12 to 16.81), and b\* which was more yellowish (65.49 to 104.5), after 30 days of storage.

The film incorporated with EO extracted from *C. zeylanicum* was quite stable, with no significant changes over 30 days for all color parameters, as can be seen in Table 1. As EOs are organic compounds, they can undergo autoxidation in the presence of energy (heat or light irradiation), leading to degradation of some compounds, as reported by Guimarães *et al.* (2008). Furthermore, whey protein has about 1.5% lipids (Ramos *et al.*, 2013), which can also undergo oxidation.

Cinnamon and rosemary EOs exhibit potent antioxidant capacity as reported by Sacchetti *et al.* (2005) and Shan *et al.* (2005), which may be associated with the compounds cinnamaldehyde and eugenol present in cinnamon oil and  $\alpha$ -pinene, present in rosemary oil, as showed by several authors (Singh *et al.*, 2007; Ojeda-Sana *et al.*, 2013). With respect to the two cinnamon species studied by Han *et al.*

(2005), *C. cassia* EO was less effective than the *C. zeylanicum* EO. In addition, Singh *et al.* (2007) have shown eugenol as the more effective antioxidant in food, which may explain the higher color stability of the film incorporated with *C. zeylanicum* EO when compared to the film incorporated with *C. cassia* EO.

According to Guimarães *et al.* (2008), the components from the EOs can undergo degradation over time, resulting in compounds from the oxidation process. Therefore, it may be inferred that the minor color changes observed for the film incorporated with *C. zeylanicum* EO when compared with the films incorporated with *C. cassia* EO (Singh *et al.*, 2007) may be due to its higher antioxidant capacity.

## Conclusion

The development of biodegradable packaging materials is an effective tool to minimize the environmental pollution caused by synthetic packaging. Antimicrobial films incorporated with essential oils have proven to provide food safety by minimizing microbial contamination, and the *Cinnamomum cassia*-based film stood out among the films in this study. However, essential oils can undergo oxidation, and therefore the films incorporated with EO can present color changes over time. In spite of the potential application in the wrapped food protection against microbial contamination, film with cinnamon EO may have limited use due to its strong aroma.

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